The Formation of Exchangeable Sulphite from Adenosine 3'-Phosphate 5'-Sulphatophosphate in Yeast

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A new low-molecular-weight bound sulphite was found in yeast enzyme reaction systems which convert the sulphur of ^{35}S -labelled adenosine 3'-phosphate 5'-sulphatophosphate into exchangeable radioactive sulphite. This bound sulphite was separated from other components by paper electrophoresis and Sephadex G-25 chromatography, and shown to be a peptide with multiple thiol groups and an estimated mol.wt. of 1400. The labelled sulphur in this peptide is highly exchangeable with unlabelled sulphite, but exchangeability decreases with time and freeze-drying. The low-molecular-weight acceptor is tightly bound to enzyme B of the yeast system and, apparently, accepts the sulphy group of adenosine 3'-phosphate 5'-sulphatophosphate and is released as bound sulphite only in the presence of enzymically or chemically reduced fraction C. It is proposed that the low-molecular-weight acceptor is a carrier peptide which, after release of the reduced sulphur, becomes re-oxidized and returns to enzyme B. Fraction C appears to function as an obligatory reductant of the oxidized acceptor before it can accept another $-\text{SO}_3^-$ moiety from adenosine 3'-phosphate 5'-sulphatophosphate. These findings are consistent with mechanisms proposed for sulphate reduction in spinach and *Chlorella*, and suggest that fraction C is the natural thiol required in these systems. An improved column technique for the preparation of adenosine 3'-phosphate 5'-sulphatophosphate is described.

Although adenosine 3'-phosphate 5'-sulphatophosphate (active sulphate) is known to be the substrate for the enzymic reduction of sulphate in yeast (Wilson, 1962), the mechanism by which this substrate is utilized has remained unclear. A yeast enzyme system, which catalyses the conversion of the sulphur of adenosine 3'-phosphate 5'-sulphatophosphate into a form that is exchangeable with sulphite, was resolved into at least three proteins: enzyme A, enzyme B and fraction C (Wilson *et al.*, 1961). Enzyme A was partially purified (Asahi *et al.*, 1961) and shown to be a protein disulphide reductase catalysing the reduction of fraction C to a protein dithiol:

An additional unidentified factor found in crude preparations of fraction C greatly stimulated this reaction. Fraction C and enzyme A appear to be identical with thioredoxin and thioredoxin reductase (NADPH-oxidized-thioredoxin oxidoreductase, EC 1.6.4.5) of *Escherichia coli* and yeast (Porque *et al.*, 1970).

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In the complete system, the sulphur of ^{35}S -labelled adenosine 3'-phosphate 5'-sulphatophosphate is converted into a 'bound sulphite', $\text{X-}^{35}\text{SO}_3^-$ (Torii & Bandurski, 1967). This reaction is assumed to involve reduced fraction C and be catalysed by enzyme B:

The bound sulphite, believed to be sulphite bound to fraction C, is a non-diffusible protein that migrates near the origin on paper electrophoresis at pH 4.5 and releases acid-volatile radioactivity ($^{35}\text{SO}_2$) only after exchange with unlabelled sulphite (eqns. 3 and 4), strongly suggesting the presence of an $-\text{S-}^{35}\text{SO}_3^-$ linkage.

The bound sulphite appeared to be an intermediate in sulphate reduction, since it was readily reduced to sulphide in the presence of NADPH and a crude yeast enzyme system.

Studies with extracts of spinach and partially purified enzymes of *Chlorella* have confirmed that

bound rather than free sulphite is formed during sulphate reduction (Schmidt & Schwenn, 1971; Abrams & Schiff, 1973; Schmidt, 1973; Schmidt *et al.*, 1974). Adenosine 5'-sulphatophosphate has been identified as the substrate reduced in these organisms (Tsang *et al.*, 1971; Schmidt, 1972; Schmidt, 1975) and adenosine 5'-sulphatophosphate sulphotransferase has been shown to transfer the sulphur of this substrate to a variety of thiol acceptors; *S*-sulphogluthathione (GSSO_3^-) is formed in the presence of reduced glutathione (Schmidt, 1972; Abrams & Schiff, 1973). The spinach and *Chlorella* studies suggest that the sulphur of adenosine 5'-sulphatophosphate is bound to a small carrier molecule during reduction to sulphide *in vivo*.

In the present paper we report the discovery of a low-molecular-weight sulphite acceptor which may be a carrier of sulphite in yeast. We have also obtained evidence that fraction C functions in the reduction of the low-molecular-weight acceptor before the transfer of the $-\text{SO}_3^-$ moiety from adenosine 3'-phosphate 5'-sulphatophosphate.

Materials and Methods

Chemicals

ATP, 3'-AMP, glucose 6-phosphate, FAD (grade III), NADP^+ (monosodium salt), NADPH (tetrasodium salt, type II) and crystalline bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] and dithiothreitol from Calbiochem, La Jolla, CA, U.S.A.; Sephadex G-25, G-50, and DEAE-Sephadex A25 from Pharmacia Fine Chemicals Inc., Piscataway, NJ, U.S.A.; bacitracin from Schwartz/Mann, Orangeville, NY, U.S.A.; 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, both scintillation grade, from Research Products International Corp., Oak Grove Village, IL, U.S.A.; and $\text{Na}_2^{35}\text{SO}_4$ (1 Ci/mmol) from New England Nuclear Corp., Boston, MA, U.S.A. Analytical-grade solvents and inorganic chemicals were used. Buffers were prepared by adjusting solutions of Tris base and acetic acid with HCl and NaOH respectively, and diluting to 0.1 M.

Enzymes

All enzymes were prepared from starch-free baker's yeast (*Saccharomyces cerevisiae*) purchased locally from Anheuser-Busch, St. Louis, MO, U.S.A. Partially purified components of the yeast sulphate-reducing system (enzyme A, enzyme B, fraction C-I and C-II) were prepared and used as previously described (Wilson *et al.*, 1961). Enzyme A contained enough glucose 6-phosphate dehydrogenase activity to catalyse the reduction of NADP^+ in the sulphotransferase and bound-sulphite assays. Protein was

determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as standard. Fraction C-III was prepared from fraction C-II by chromatography on a column (1.3 cm $\times$ 109 cm) of Sephadex G-50. Fraction C-II (50–90 mg) was placed on the column and eluted with 0.1 M-sodium acetate buffer, pH 5.0. Active fractions were determined by measuring the enzymic formation of SH (see thiol assays). Active material was dialysed against 0.001 M-Tris/HCl/0.001 M-EDTA, pH 7.5, and freeze-dried (12–20 mg).

Adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate

This substrate was prepared from the diethyl ether-pretreated sulphate-activating system of Robbins & Lipmann (1958) by an improved column technique. The incubation mixture (20 ml) contained (in μmol): Tris/HCl, pH 8, 1000; MgCl_2 , 200; ATP, 200; 3'-AMP, 400; $\text{Na}_2^{35}\text{SO}_4$, 400 (1600 μCi); enzyme (dialysed for 16 h), 120 mg. Incubation was for 180 min at 37°C. The reaction was stopped by immersing the reaction flask in a boiling-water bath for 3 min. Denatured protein was removed by centrifugation. The adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate was purified by chromatography on a column (2.6 cm $\times$ 35 cm) of DEAE-Sephadex A-25 suspended in 0.05 M-ammonium formate and eluted with a volatile buffer used by others (Balasubramanian *et al.*, 1967; Adams *et al.*, 1971) with a linear gradient from 0.05 M- to 0.85 M- NH_4HCO_3 with 1.8 litres in each flask and a flow rate of 2.5 ml/min. Samples of the fractions were monitored for radioactivity by means of a gas-flow planchet counter (Nuclear-Chicago, Des Plaines, IL, U.S.A.) for nucleotides by E_{259} , and for NH_4HCO_3 concentration by Nessler's reagent. The ^{35}S -labelled nucleotide was eluted from the column at approximately 0.6 M- NH_4HCO_3 , and identified by paper electrophoresis in 0.1 M-sodium acetate buffer, pH 4.5. The fractions containing the adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate were combined and freeze-dried until the water and volatile buffer were gone. A total of 2.18 μmol of product (4 $\mu\text{Ci}/\mu\text{mol}$) was recovered in this preparation (Fig. 1). By using a steeper gradient (0.1–1.0 M- NH_4HCO_3) with 1.5 litres in each flask and fresh undialysed enzyme, the yield was increased to 14.4 μmol (1.8 $\mu\text{Ci}/\mu\text{mol}$). During freeze-drying, however, some breakdown occurred, and 80% of the radioactivity in the final product was in adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate and 20% in $^{35}\text{SO}_4^{2-}$. The product, resuspended in water and frozen, remained stable for several months.

Sulphotransferase assay

Total exchangeable radioactive sulphite formed from adenosine 3'-phosphate 5'-[^{35}S]sulphatophos-

Fig. 1. Elution profile of adenosine 5'-[^{35}S]sulphatophosphate and adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate from DEAE-Sephadex A-25

Adenine nucleotides (—) as measured at 259 nm in 1 cm cells, ^{35}S radioactivity (···) in c.p.m./10 μl sample, and concentration of NH_4HCO_3 eluting buffer (---) in mol/l. For conditions of chromatography, see the Materials and Methods section. 'APS' and 'PAPS' indicate adenosine 5'-sulphatophosphate and adenosine 3'-phosphate 5'-sulphatophosphate respectively. The fraction size was 10 ml.

phate (eqn. 2) is used as a measure of enzyme B and fraction C activity. Although the exact nature of the sulphy-group acceptor is not known, the experimental evidence in this paper demonstrates that enzyme B is directly involved in the transfer of the $-\text{SO}_3^-$ moiety of adenosine 3'-phosphate 5'-sulphatophosphate to a carrier peptide in which the labelled sulphur is completely exchangeable with unlabelled sulphite. Thus enzyme B has been termed a sulphotransferase in agreement with the terminology of others (Schmidt, 1972; Abrams & Schiff, 1973), although it differs markedly from all other sulphotransferases in which adenosine 3'-phosphate 5'-sulphatophosphate is known to be the sulphur donor (Roy & Trudinger, 1970).

The sulphotransferase assay is adapted from that of Wilson *et al.* (1961) and is carried out in 10 ml flasks with serum caps and glass cups (10 mm $\times$ 15 mm) suspended from the caps. The basic incubation mixture (0.75 ml) contained in μmol : Tris/HCl, pH 7.5, 25; MgCl_2 , 2.5; EDTA, 1; glucose 6-phosphate, 2.5; NADP^+ , 0.25; FAD, 0.006; NaF, 50; adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate, 0.144 (1–2 $\mu\text{Ci}/\mu\text{mol}$); enzyme A, 0.11 mg; enzyme B, 0.15 mg; fraction C-I, C-II or C-III, 0.5–8 mg. Unlabelled sulphite (2.5 μmol) was present during the incubation or added at the end of the incubation. The flasks were alternately flushed with argon and evacuated and then incubated for 1 or 2 h at 37°C under vacuum. The reaction was terminated by the addition of 0.5 ml of 5% (w/v) H_3PO_4 in 2.4% (w/v)

mannitol by syringe. The volatile sulphite was collected in 0.10 ml of 0.15 M-NaOH/0.001 M-EDTA in the glass cups during a 90 min diffusion period at 37°C. Samples from these cups were used to determine acid-volatile radioactivity ($^{35}\text{SO}_2$) in a liquid-scintillation spectrometer (Packard Tricarb model 2000) and sulphite recovery by the fuchsin assay (Grant, 1947). Recovery of unlabelled sulphite was used to calculate total $^{35}\text{SO}_2$ formation as previously described (Wilson *et al.*, 1961). The liquid-scintillation mixture contained 100 g of naphthalene, 5 g of 2,5-diphenyloxazole, 1.5 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 730 ml of dioxan, 125 ml of toluene, and 35 ml of methanol.

Assay for protein- SSO_3^- and other forms of bound sulphite

The same general incubation mixture and conditions were used as in the sulphotransferase assay, but unlabelled sulphite was omitted and various amounts of enzyme and reagents were used. At the end of the incubation the flasks were held at 80°C for 5 min or placed on ice, and 0.1 ml samples were transferred immediately to paper electrophoresis strips. Electrophoresis was carried out on acid-washed Whatman 3MM paper on an EC Apparatus Co. flat-bed (21 cm $\times$ 45 cm) unit for 5–7 h at 10 V/cm. Radioactive areas were detected by scanning dried electrophoresis strips on a Packard model 7201 radiochromatogram scanner. Protein- SSO_3^- mi-

grated at 0.65 times the rate of sulphate at pH 4.5 in 0.1 M-sodium acetate buffer. Estimates of the concentration of protein-SSO₃⁻ and other radioactive components were made by visual comparisons and by estimating the areas under the various peaks. The sulphite-exchangeable radioactivity of each eluted product was determined by an adaptation of the sulphotransferase assay. Samples were placed in flasks with 100 μ mol of Tris/HCl, pH 7.6, 2 μ mol of EDTA and 2.5 μ mol of Na₂SO₃ in a total volume of 0.75 ml. The flasks were evacuated, allowed to exchange for 10 min at 37°C and the acid-volatile ³⁵SO₂ was determined after acidification as above.

Assays for thiol groups and reducible disulphide

Thiol groups were determined colorimetrically at 412 nm by the use of Ellman's reagent and a molar extinction coefficient of 13 600 litre·mol⁻¹·cm⁻¹ (Ellman, 1959). Total protein SH was determined in the presence of 0.5% sodium dodecyl sulphate by the method of Sedlak & Lindsay (1968). Enzymically formed thiol or reducible disulphide was determined as previously described (Wilson *et al.*, 1961) in a modified incubation mixture (1 ml) containing (in μ mol): K₂HPO₄, pH 7.50; EDTA, 1; NADPH, 0.1; enzyme A, 0.11 mg; sample; Ellman's reagent, 0.1 μ mol in Tris/HCl (200 μ mol), pH 8.

Results

Preparation of adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate

Published methods of preparation of adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate give low enzymic activities, a variable mixture of products, or poor recovery. The method described here (see the Materials and Methods section) yields micromolar quantities of this important substrate after purification by Sephadex chromatography. 3'-AMP was included in the reaction mixture to protect the product from the activity of 3'-nucleotidases (Klein & Schoenhard, 1970). The adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate, completely separated from adenosine 5'-[³⁵S]sulphatophosphate and other nucleotides by elution from DEAE Sephadex A-25 with NH₄HCO₃ (Fig. 1), was then freeze-dried to remove water and the volatile buffer.

Total exchangeable radioactive sulphite

Purification of fraction C. The final step in the procedure for the purification of fraction C (Wilson *et al.*, 1961) has been changed to a column step to provide larger amounts than could be obtained by paper electrophoresis. Fraction C-III was prepared

from fraction C-II by chromatography on Sephadex G-50 columns. Reducible disulphide fractions were located by the enzymic thiol assay. The need for concentrated fractions for the assays and the presence of several fraction C-like compounds complicated the column studies. By overloading the columns it was possible to obtain individual fractions concentrated enough to permit definitive thiol assays (Fig. 2). Fractions from the (a), (b) and (c) areas of the column were combined, freeze-dried and assayed by the sulphotransferase assay. The major peak of fraction C activity coincided with the major thiol peak. The high-molecular-weight material (a) was completely inactive, despite a substantial concentration of thiol. The dependence of the sulphotransferase reaction on fraction C and the specific activity of fraction C at various stages of purification is shown in Table 1. A fivefold purification and about 10% recovery of activity were achieved.

Participation of reduced fraction C. Several experiments suggest that reduced fraction C is involved in the formation of sulphite-exchangeable radioactivity from adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate (eqns. 2-4). Dithiothreitol can reduce fraction C and substitute for enzyme A and the

Fig. 2. Gel chromatography of fraction C-II on Sephadex G-50

Protein (■) as measured at 280 nm in a 1 cm cell, thiol (●) determined enzymically, and exchangeable radioactive sulphite (▲) determined in sulphotransferase assays of fractions designated as (a), (b) and (c). Assays were as described in the Materials and Methods section. Fraction C-II (90 mg) was placed on a column (1.3 cm × 114 cm) of Sephadex G-50 and eluted with 0.1 M-sodium acetate buffer, pH 5. The fraction size was 2 ml.

Table 1. *Purification of fraction C*

The activities were determined in routine 1 h sulphotransferase assays (see the Materials and Methods section). A unit of fraction C activity is defined as the amount of fraction C that catalyses the formation of 1 nmol of $^{35}\text{SO}_2/\text{h}$ in the standard assay. The specific activity is defined as the number of fraction C units/mg of protein. Fraction C-I was prepared from 240 g of acetone-treated yeast powder.

Assay	Added fraction C	$^{35}\text{SO}_2/\text{assay}$ (nmol)	Specific activity (units/mg of protein)	Total protein (mg)	Total activity (fraction C units)
1	None	0.16	—	—	—
	Fraction C-I (8 mg)	8.59	1.05	1380	1449
	Fraction C-II (4 mg)	4.32	1.04	208	216
2	None	0.76	—	—	—
	Fraction C-III (2 mg)	10.46	4.85	30	146

Table 2. *Participation of fraction C in the formation of exchangeable radioactive sulphite*

Exchangeable radioactive sulphite was determined in 1 h incubation sulphotransferase assays with various reducing systems. Enzyme A and NADP^+ were replaced with 1 μmol of dithiothreitol where indicated. Fraction C-III-T was reduced with dithiothreitol, eluted in the void volume of a Sephadex G-25 column (Fig. 3) and concentrated by freeze-drying before assay.

Added fraction C	$^{35}\text{SO}_2$ formed in various reducing systems		
	NADP^+ +enzyme A (nmol)	Dithiothreitol (nmol)	None (nmol)
None	0.49	0.51	—
Fraction C-III (2.4 mg)	2.35	2.83	0.05
Fraction C-III-T (2.4 mg)	2.52	—	0.07

NADPH -generating system (Table 2). The dithiothreitol system generally shows a slight stimulation over the enzyme A– NADPH system, but dithiothreitol cannot substitute for fraction C, at least when small amounts of enzyme B are used. The control values with enzyme B alone suggest that enzyme B contains traces of fraction C.

Attempts were made to carry out the sulphotransferase reaction with chemically reduced fraction C. Fraction C-III was treated with dithiothreitol under aerobic conditions on a Sephadex G-25 column. Although untreated fraction C-III appeared in a single peak in the void volume of a control column, the dithiothreitol-treated fraction C-III separated into a number of low-molecular-weight fractions (Fig. 3), indicating the presence of mixed disulphides in the original preparation. However, as shown in Fig. 3, most of the thiol-containing material at the time of assay (3 h after elution) was found in the void volume and the dithiothreitol peak. The material in the void volume, designated as fraction C-III(T), was concentrated by freeze-drying and tested in a usual sulphotransferase assay with and without the enzyme A– NADPH reducing system. Although the treated material was fully active in the presence of the reducing system, it, like the original fraction C-III(T), was inactive in the absence of a

reducing system (bottom of Table 2). These results indicate that dithiothreitol-reduced fraction C had probably been re-oxidized during freeze-drying. The complete lack of $^{35}\text{SO}_2$ formation in the absence of a reducing system clearly indicates that reduced fraction C is required before sulphite-exchangeable radioactivity is formed from adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate.

Participation of enzyme B. Careful analysis of assays with fraction C suggested that the concentration of enzyme B was limiting in the standard sulphotransferase assay system. This was confirmed when enzyme B was assayed over a sixfold range of concentrations with the enzyme A– NADP^+ or dithiothreitol reducing systems (Fig. 4). The non-linear increase in activity becomes a straight line when the activity with dithiothreitol is plotted against the square of the enzyme concentration (Fig. 4, insert), suggesting that a dissociable cofactor is present and required in the reaction (Dixon & Webb, 1964).

Bound-sulphite reaction products

In the experiments described above, unlabelled sulphite was present to exchange with any radioactive bound sulphite. In the following experiments unlabelled sulphite was omitted from the incubation

Fig. 3. Elution profile of dithiothreitol-reduced fraction C-III from a Sephadex G-25 column

Fraction C-III (12mg), 50 μ mol of phosphate buffer, pH7, and 1 μ mol of EDTA in a volume of 0.47 ml were treated with 1.2 μ mol of dithiothreitol for 10min at 30°C. The reaction mixture was placed on a column (1 cm $\times$ 20 cm) of Sephadex G-25, and 1–2 ml fractions were eluted with 0.05 M-sodium acetate/1 mM-EDTA, pH4.5. Protein (—) was determined by measurements at 276 nm in 1 cm cells and thiol (· · ·) by assay of samples with Ellman's reagent at 412 nm in 1 cm cells.

mixture so that radioactive intermediates could accumulate. In some experiments, after samples had been removed for analysis, unlabelled sulphite was added, allowed to exchange for 30min, and then assayed for exchangeable radioactive sulphite in the usual way.

Discovery and characterization of the 'bound sulphite', protein-SSO₃⁻. A major peak of sulphite-exchangeable radioactivity was observed when reaction mixtures were electrophoretically separated. This new peak of material appeared between adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate and ³⁵SO₄²⁻ in the electrophoretograms of a variety of reaction mixtures (Fig. 5). The radioactive material in the new peak was ninhydrin-positive and, after elution with water and exchange with unlabelled sulphite was found to be 80–100% acid-volatile. The radioactive unknown material ran as a single peak through Sephadex-G25, and was retarded to the same extent as bacitracin which has a mol.wt. of 1450 (Fig. 6a). Preliminary amino acid analysis of the two preparations indicated the presence of glutamic acid, aspartic acid, serine, glycine, alanine and histidine.

These results suggested the presence of a small -S-sulphoprotein or peptide similar to those pre-

viously described in yeast (Torii & Bandurski, 1967), spinach (Schmidt & Schwenn, 1971) and *Chlorella* (Abrams & Schiff, 1973). The new material, tentatively designated as protein-SSO₃⁻, is strongly anionic, as demonstrated by its electrophoretic mobility, and readily diffuses through a dialysis bag in 0.01 M-Tris/HCl/0.001 M-EDTA at pH7.5, in contrast with the bound sulphite of yeast described above. It is relatively stable to freezing and thawing, but lost 40% of its activity after 5min at 80°C (Table 3). It also remained relatively stable on dry electrophoresis paper for 1 week.

In some preparations of protein-SSO₃⁻, however, a double peak of radioactivity occurred during Sephadex G-25 chromatography (Fig. 6b) together with a marked decrease in sulphite-exchangeable radioactivity. This occurred during attempts to obtain larger amounts of material by the same purification procedure (electrophoresis, elution, freeze-drying, chromatography). Further, all preparations contained variable amounts of thiol (Table 4) and reducible disulphide. Since sulphur-containing compounds are known to be unstable, it seems likely that oxidation and complexing by disulphide formation during purification may ac-

Fig. 4. Effect of enzyme B concentration on the formation of exchangeable radioactive sulphite

Exchangeable radioactive sulphite ($^{35}\text{SO}_2/\text{flask}$) was determined in 1 h incubation sulphotransferase assays (see the Materials and Methods section) without added fraction C in two reducing systems, enzyme A + NADP $^+$ (○) and dithiothreitol (●). Enzyme A has glucose 6-phosphate dehydrogenase activity and enzyme B has some fraction C activity. The insert shows the straight line obtained when the activity with dithiothreitol is plotted against the square of enzyme concentration.

count for the higher-molecular-weight material and the decreased volatility in the double peaks.

Formation of protein-SSO $_3^-$. The protein-SSO $_3^-$ is detectable in the reaction mixture within 15 min and continues to increase until adenosine 3'-phosphate 5'-sulphatophosphate is depleted (Fig. 7, Table 5). The material is formed aerobically or anaerobically in approximately equal amounts (Table 5) under a wide variety of conditions. The basic requirements for its formation are adenosine 3'-phosphate 5'-sulphatophosphate, enzyme B and a disulphide-reducing system. At low concentrations of enzyme B, the reducing system may be enzyme A, NADP $^+$ and fraction C, or dithiothreitol and fraction C (Figs. 5a and b). At higher concentrations of enzyme B, fraction C may be omitted (Figs. 5c and 5d).

No detectable amounts of protein-SSO $_3^-$ are obtained in the absence of one of these reducing systems. These requirements are identical with those for the conversion of the sulphur of adenosine 3'-phosphate 5'-sulphatophosphate into exchangeable radioactive sulphite in the sulphotransferase assay (Table 2, Fig. 4). These and other data suggest that protein-SSO $_3^-$ is accumulating and that fraction C is recycling in this system. Enzyme B, at higher concentrations, presumably contains sufficient fraction C for this purpose.

After prolonged storage of enzyme B, fraction C-I or C-II and increasing amounts of enzyme B are required to obtain the amounts of protein-SSO $_3^-$ needed for characterization and analysis (Table 6).

Since fluoride was used by Torii & Bandurski

Fig. 5. Electrophoretograms showing formation of protein- SSO_3^- in four reaction mixtures

Radioactivity of electrophoresis strips containing four reaction mixtures after electrophoresis for 4 h with 0.1 M-sodium acetate buffer, pH 4.5 at 15°C. All reaction mixtures (0.75 ml) contained in μmol : Tris/HCl, pH 7.6, 25; MgCl_2 , 2.5; EDTA, 1; glucose 6-phosphate, 2.5; FAD, 0.006; NaF, 50; adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate (1.38 $\mu\text{Ci}/\mu\text{mol}$), 0.144. In addition (a) included enzyme B, 0.15 mg; fraction C-I, 8 mg; enzyme A, 0.11 mg; NADP^+ , 0.25 μmol ; (b) included enzyme B, 0.15 mg; fraction C-I, 8 mg; dithiothreitol, 1 μmol ; (c) included enzyme B, 0.9 mg; enzyme A, 0.11 mg; NADP^+ , 0.25 μmol ; (d) included enzyme B, 0.9 mg; dithiothreitol, 1 μmol . Abbreviation = PAPS, adenosine 3'-phosphate 5'-sulphatophosphate.

(1967) to improve the yields of bound sulphite from adenosine 3'-phosphate 5'-sulphatophosphate, experiments were carried out to determine the effect of F^- on protein- SSO_3^- formation and the formation of exchangeable sulphite in the sulphotransferase assay. A tenfold decrease in the amount of F^- added

did not affect the amount of radioactive sulphite formed or of protein- SSO_3^- eluted, but omission of F^- decreased the amounts of both products by one-third (Table 7). The removal of Mg^{2+} from the basic minus F^- incubation mixture strongly stimulated radioactive sulphite and protein- SSO_3^- forma-

Fig. 6. Gel chromatography of protein-SSO₃⁻ on Sephadex G-25, showing single and double peaks of radioactivity

In (a) a total of 9.1 nmol of protein-SSO₃⁻ (18180 c.p.m.) eluted from electrophoresis strips was placed on a column (1 cm $\times$ 100 cm) of Sephadex G-25 and eluted in 2 ml fractions with 0.05 M acetic acid at a rate of 10 ml/h. Protein-SSO₃⁻ ($\circ$) was determined by liquid-scintillation counting of 0.10 ml samples. The column had previously been calibrated under identical conditions with 3 mg of Blue Dextran (mol.wt. 2×10^6) measured at 412 nm ($\bullet$) and 4 mg of bacitracin (mol.wt. 1450) measured at 280 nm ($\blacksquare$), both with 1 cm cells. In (b), the eluted and concentrated protein-SSO₃⁻ (0.63 μ Ci/ μ mol) from 0.675 ml of a reaction mixture was applied to the same column and eluted as above. The fractions under the two peaks were combined and designated as preparations 5a and 5b (see Table 4).

Table 3. Stability and volatility of protein-SSO₃⁻ and adenosine 3'-phosphate 5'-sulphatophosphate

Three reaction flasks were used, containing the reaction mixture used in Fig. 5(c). The mixture in the third flask was boiled before incubation. Samples from the first and third flasks were applied directly to electrophoresis strips at the end of the 1 h incubation; the other mixture was held at 80°C for 5 min before a sample was applied and electrophoresis initiated. The remainder of each mixture was frozen for 2 days, thawed, and fresh samples were subjected to electrophoresis. All sample volumes were 0.10 ml. The adenosine 3'-phosphate 5'-sulphatophosphate or active sulphate areas were eluted from the two strips containing the boiled enzyme mixture and the protein-SSO₃⁻ areas were eluted from the other strips. The total radioactivity and the sulphite-exchangeable volatile radioactivity were determined as described in the Materials and Methods section.

Treatment of reaction mixture	Eluted material	Radioactivity and volatility of eluted material/electrophoresis strip		
		Total ³⁵ S (nmol)	Volatile ³⁵ S (nmol)	Volatility (%)
Immediate assay				
No treatment	Protein-SSO ₃ ⁻	1.10	0.88	80
5 min 80°C post-incubation	Protein-SSO ₃ ⁻	0.62	0.53	84
2 min 100°C pre-incubation	Active sulphate	11.52	0.01	0.1
Frozen 2 days				
No other treatment	Protein-SSO ₃ ⁻	1.70	1.34	79
5 min 80°C post-incubation	Protein-SSO ₃ ⁻	1.13	0.90	79
2 min 100°C pre-incubation	Active sulphate	9.57	0.00	0

tion and demonstrated that Mg^{2+} was not required for the formation of either product (Table 7). In this experiment, the substitution of dithiothreitol for enzyme A and NADP^+ resulted in a marked loss in both activities. However, the observed activities were sufficient to indicate that the dithiothreitol-stimulated formation of radioactive sulphite and protein- SSO_3^- also lacks a requirement for Mg^{2+} . Visual inspection of the electrophoretograms for this experiment showed that the presence of Mg^{2+} in these minus- F^- mixtures was correlated with disappearance of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate and an increase in $^{35}\text{SO}_4^{2-}$. These experiments suggest that a Mg^{2+} -activated sulphatase is present which is inhibited by F^- .

Other forms of 'bound sulphite'. Protein- SSO_3^- formation and the conversion of adenosine 3'-phosphate 5'-sulphatophosphate into exchangeable sulphite are directly compared in the fluoride experiment (Table 7). The data show that approximately one-half of the radioactive sulphite formed in this 2 h incubation assay appears in protein- SSO_3^- .

Two other minor peaks of sulphite-exchangeable radioactivity have been observed on electrophoresis strips (Table 8). One occurs near the origin (see Fig. 7). Its position and volatility (approximately 50%) indicate that it is the 'bound sulphite' of yeast described previously (Torii & Bandurski, 1967). Under our

conditions it is normally present in small amounts and is not detectable during the first 30 min of incubation. The other area of exchangeable radioactivity is present in the leading edge of the adenosine 3'-phosphate 5'-sulphatophosphate peak (Fig. 7 and Table 8).

Table 4. *Thiol content of protein- SSO_3^- preparations from Sephadex G-25 columns*

Total ^{35}S and total thiol were determined on samples of each purified preparation as described in the Materials and Methods section. Preparation 2 was derived from the reaction mixture described in Fig. 5(c). Preparations 4 and 5 were derived from an incubation mixture (0.75 ml) containing in μmol : Tris/HCl, pH 7.6, 50; MgCl_2 , 5; EDTA, 2; glucose 6-phosphate, 5; NADP^+ , 0.5; FAD, 0.012; NaF, 100; adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate ($1 \mu\text{Ci}/\mu\text{mol}$), 0.144; enzyme A, 0.22 mg; enzyme B, 1.8 mg; fraction C, 8 mg. Preparations 5a and 5b represent material eluted separately from the double peaks of radioactivity shown in Fig. 6(b).

Protein- SSO_3^- preparation	Total ^{35}S (nmol)	Total thiol (nmol)
2	6.51	302
4	25.21	52
5a	17.09	29
5b	6.04	70

Fig. 7. *Electrophoretograms showing enzymic formation of protein- SSO_3^- from adenosine 3'-phosphate 5'-sulphatophosphate*

The radioactivity of electrophoresis strips containing reaction mixtures incubated for (a) 15 min and (b) 120 min was measured. Incubation mixtures contained double the amounts of all components used in Fig. 5(c). Electrophoresis was carried out on Whatman 3 MM paper at 10 V/cm with 0.1 M-sodium acetate buffer, pH 4.5 for 7.5 h at 13°C. Compounds shown in Fig. 7(b) include a compound near the origin at 2 cm, adenosine 3'-phosphate 5'-sulphatophosphate or active sulphate at 27 cm, and protein- SSO_3^- at 35 cm.

Isolation of protein-SSO₃⁻. Paper electrophoresis with acetate buffer at pH4.5 has provided the best initial method of separation of protein-SSO₃⁻ from products of the incubation mixture (Fig. 7). Adenosine 3'-phosphate 5'-sulphatophosphate and protein-SSO₃⁻ migrate closely together in citrate buffer at pH5.5 and in Tris/HCl buffer at pH7.6. Satisfactory separation at pH7.6 was achieved, however, on a high-voltage apparatus (Savant) with a longer bed length (Fig. 8). This separation demonstrates that protein-SSO₃⁻ occurs in the neutral reaction mixture as well as in acetate buffer at pH4.5.

Elution from paper-electrophoresis strips, concentration by freeze-drying, and passage through a Sephadex G-25 column have so far provided the only satisfactory purification steps, although the method is time-consuming and results in a loss in volatility.

Table 5. *Time-course for protein-SSO₃⁻ formation under aerobic and anaerobic conditions*

Reaction mixtures and electrophoresis as in Fig. 7 except that the aerobic set of flasks was left uncapped during incubation. At the indicated times the flasks were removed and held at 0°C until 0.1ml samples were applied to electrophoresis paper. The relative radioactivity of protein-SSO₃⁻ formed during each treatment was calculated after determining the area obtained under each peak with identical settings of the radiochromatogram scanner.

Time of incubation (min)	Total protein-SSO ₃ ⁻ formed/sample	
	Aerobically (c.p.m./peak)	Anaerobically (c.p.m./peak)
15	412	380
30	611	806
60	1520	2074
120	2258	2313

Table 6. *Activity of enzyme B and fraction C in the formation of exchangeable radioactive sulphite and protein-SSO₃⁻*

Mixtures were incubated for 1 h without sulphite. Mixtures (0.75 ml) contained in μ mol: Tris/HCl, pH7.5, 50; MgCl₂, 5; EDTA, 2; glucose 6-phosphate, 5; NADP⁺, 0.5; FAD, 0.012; NaF, 100; adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate (1-2 μ Ci/ μ mol), 0.144; enzyme A, 0.22mg; enzyme B and fraction C-I or C-II as indicated. After removal of 0.10ml samples for electrophoresis, sulphite was added and the remaining reaction mixture assayed for total exchangeable radioactive sulphite as described in the Materials and Methods section. Estimates of protein-SSO₃⁻ formation are based on comparisons of radioactivity scans of electrophoresis strips obtained and counted for radioactivity under identical conditions. Electrophoresis was at pH4.5 for 6 h at 5°C. Aged enzyme was frozen for 8 months and thawed several times.

Addition (mg)	Total exchangeable radioactive sulphite/flask (nmol)	Protein-SSO ₃ ⁻ formation by visual comparison (estimate)
Aged enzyme B (0.9)	7.7	—
Aged enzyme B (0.9)+fraction C-II (1)	8.4	—
Aged enzyme B (0.9)+fraction C-I (8)	17.4	+
Aged enzyme B (1.8)	31.8	+
Aged enzyme B (1.8)+fraction C-II (0.5)	42.0	+
Fresh enzyme B (1.8)	—	+
Fresh enzyme B (1.8)+fraction C-I (8)	—	+++

Table 7. *Effect of F⁻ and Mg²⁺ on the formation of exchangeable radioactive sulphite and protein-SSO₃⁻*

Mixtures incubated for 2 h without sulphite. Mixtures (0.75 ml) contained in μ mol: Tris/HCl, pH7.6, 50; EDTA, 2; glucose 6-phosphate, 5; FAD, 0.012; adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate (0.54 μ Ci/ μ mol), 0.144; NADP⁺, 0.5; enzyme A, 0.22mg; enzyme B, 1.8mg; where indicated, MgCl₂, 5; NaF, 10 or 100; dithiothreitol, 1. Enzyme A and NADP⁺ were omitted when dithiothreitol was added. Samples were removed for electrophoresis, unlabelled sulphite was added, and assays for total exchangeable radioactive sulphite and protein-SSO₃⁻ (visual) were carried out as described in Table 6. The radioactivity of eluted samples of protein-SSO₃⁻ was used to calculate protein-SSO₃⁻ per flask. Electrophoresis was carried out at pH4.5 for 5.5 h at 5°C.

Addition or omission (μ mol)	Total exchangeable radioactive sulphite/flask (nmol)	Protein-SSO ₃ ⁻ formation	
		Per flask (nmol)	Visual (estimate)
+MgCl ₂ , +NaF (100)	63.5	30.6	++
+MgCl ₂ , +NaF (10)	61.1	33.6	++
+MgCl ₂ , -NaF	42.2	19.0	+
-NaF, +MgCl ₂	31.6	—	+
-NaF, -MgCl ₂	46.0	—	++
-NaF, -MgCl ₂ , +dithiothreitol	16.5	—	+

Table 8. Properties of three forms of 'bound sulphite' eluted from a single electrophoresis strip

These preparations were eluted from the strip shown in Fig. 7(b) from the areas indicated. The relative migration indicates the migration of the mid-point of each peak relative to radioactive sulphate. The total radioactivity and the sulphite-exchangeable volatile radioactivity are expressed per 0.10 ml sample placed on the electrophoresis strip. In other assays, as in Table 3, adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate (^{35}S -labelled active sulphate) and radioactive sulphate were shown to have no sulphite-exchangeable volatile radioactivity.

Designation of eluted area	Relative migration	Total ^{35}S (nmol)	Volatile ^{35}S (nmol)	Volatility (%)
Origin compound (2cm)	0.04	0.78	0.34	44
Active sulphate (27cm)	0.50	—	—	—
Active sulphate 29cm)	0.54	0.88	0.06	7
Protein-SSO $_3^-$ (35cm)	0.65	11.70	6.89	59
Sulphate (not shown)	1.00	—	—	—

Fig. 8. Electrophoretograms showing formation of protein-SSO $_3^-$ at pH 7.6 with various amounts of fluoride

Electrophoresis of samples (0.1 ml) from the reaction mixtures of Table 7 were carried out on a Savant high-voltage unit with a flat bed (76 cm) at 23 V/cm with 0.1 M-Tris/HCl, pH 7.6 at 17°C. Abbreviation: PAPS, adenosine 3'-phosphate 5'-sulphatophosphate.

The material binds irreversibly to DEAE-cellulose columns, and direct application of the reaction mixtures to Sephadex G-25 columns does not result in satisfactory separation of the reaction components.

Origin of protein-SSO $_3^-$. Although the ^{35}S -labelled sulpho group is obviously derived from adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate, there appear to be alternative sources of the protein

component of protein-SSO $_3^-$. The primary source is enzyme B, since dialysed enzyme B was the only source of protein in the simplest system in which protein-SSO $_3^-$ was formed (Fig. 5d), and enzyme B concentration studies demonstrated the presence of a dissociable cofactor (Fig. 4).

If the sulpho group is attached to a thiol group in protein-SSO $_3^-$, we would expect to find thiol or

Fig. 9. Enzymic thiol formation in enzyme B and fraction C

Preincubation was for 20 and 26 min during reduction with NADPH, and the incubation mixture was as described in the Materials and Methods section with enzyme B (0.15 mg) and fraction C-II (1 mg in *a*, or 0.5 mg in *b*) added where indicated. Ellman's reagent and Tris were added, as shown by the arrow, after the preincubation, and thiol was determined by measurements at 412 nm in 1 cm cells using a reaction mixture without enzyme as a blank. Abbreviations: Enz, enzyme; Fr, fraction.

reducible dithiol in enzyme B. Studies of the reducible disulphide bonds in enzyme A, enzyme B and fraction C are presented in Fig. 9. Fig. 9(*a*) shows that 1 mg of fraction C-II is completely reduced in a few minutes, but that thiol formation continues at a rapid rate in the presence of small amounts of enzyme B. Fig. 9(*b*) shows that enzyme B is reduced slowly, but continuously, in the absence of added fraction C. The addition of 0.5 mg of fraction C-II, however, leads to the rapid formation of additional thiol. These data demonstrate that fraction C catalyses the reduction of disulphide bonds in enzyme B. The slow reaction in the absence of added fraction C supports the earlier conclusion that enzyme B contains small amounts of fraction C.

The presence of reducible disulphide bonds in enzyme B and of thiol and reducible bonds in protein-SSO₃⁻ (Table 4) are in agreement with the evidence that the peptide portion of protein-SSO₃⁻ is derived from enzyme B. Since protein-SSO₃⁻ is dialysable, and enzyme B has been extensively dialysed, the sulphite acceptor peptide appears to be covalently linked to enzyme B. Thus the enzyme B complex is required in large amounts, since it furnishes one of the substrates for the reaction.

After loss of activity during aging, protein-SSO₃⁻ formation has been restored by the addition of increased amounts of enzyme B and by the addition of fraction C-I or C-II. The stimulation by relatively large amounts of fraction C-I (Table 6) suggests that

some of the peptide precursor of protein-SSO₃⁻ may originate from fraction C-I. Here, too, it may be covalently linked to fraction C or some other high-molecular-weight material, since fraction C-I has been extensively dialysed. If it were attached by disulphide bonds, it could, conceivably, be released by the enzyme A/NADPH/fraction C reducing system. The presence of a number of mixed disulphides in fraction C-III (Fig. 3) supports this hypothesis. Attempts to restore activity by the addition of low-molecular-weight dialysable fractions have, so far, been unsuccessful.

Discussion

The isolation from yeast of a low-molecular-weight 'bound sulphite', designated in this paper as 'protein-SSO₃⁻', confirms and clarifies the participation of a bound sulphite in the enzymic reduction of the sulphate of adenosine 3'-phosphate 5'-sulphatophosphate as demonstrated by Torii & Bandurski (1967) and others (Schmidt, 1973; Abrams & Schiff, 1973). These studies also clarify the role of fraction C in sulphate reduction and explain the stimulation of disulphide reduction by an unidentified factor (Asahi *et al.*, 1961).

Bound sulphite

Protein-SSO₃⁻ of yeast as described in the present paper, bound sulphite of yeast (Torii & Bandurski,

Scheme 1. Tentative scheme for assimilatory sulphate reduction from adenosine 3'-phosphate 5'-sulphatophosphate

See the Discussion section for details.

1967), and the sulphite carrier of *Chlorella* (Schmidt, 1973; Abrams & Schiff, 1973) have several properties in common. All accept the $-^{35}\text{SO}_3^-$ moiety of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate (adenosine 5'-[^{35}S]sulphatophosphate in the case of *Chlorella*) in the presence of the required enzyme system, and all release acid-volatile $^{35}\text{SO}_2$ only after exchange with unlabelled sulphite. However, the *Chlorella* carrier and protein- SSO_3^- are diffusible proteins with an apparent mol.wt. of 1400, whereas the original bound sulphite of yeast is non-diffusible, with an approximate mol.wt. of 10000. The present data demonstrate that the bound sulphite of yeast appears in the reaction mixture after the formation of protein- SSO_3^- . Thus it appears that the original bound sulphite was fraction C which secondarily, but reversibly (since it can be reduced to sulphide), accepts the $-\text{SO}_3^-$ moiety from protein- SSO_3^- . This is in accord with *Chlorella* studies showing that the sulpho group can be transferred non-enzymically from adenosine 5'-sulphatophosphate sulphotransferase to a number of thiol acceptors (Abrams & Schiff, 1973), and with data from yeast and *Chlorella* showing that unlabelled sulphite will not exchange with adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate in the absence of active enzyme. Thus protein- SSO_3^- and the carrier sulphite of *Chlorella* appear to be similar materials and are different from the

original higher-molecular-weight bound sulphite of yeast.

Mechanism

A unified scheme (Scheme 1) that can explain the yeast and *Chlorella* data is presented. Protein- SSO_3^- is assumed to be the sulphite carrier. It contains thiol groups and is required in substrate amounts in our system where it accumulates as an intermediate in a form such as Carrier-S-SO_3^- . In the absence of the complete reducing system, some of the bound sulphite may be transferred non-enzymically to fraction C. In the *Chlorella* system the carrier may remain bound to adenosine 5'-sulphatophosphate sulphotransferase, transfer sulphite to various thiol acceptors (Abrams & Schiff, 1973), or be transferred to the next enzyme, thiosulphonate reductase, and then reduced to sulphide in the presence of thiol and reduced ferredoxin (Schmidt, 1973; Schmidt *et al.*, 1974).

Our studies with enzyme B and fraction C suggest that, after the formation of cysteine, the oxidized carrier returns to enzyme B (or adenosine 5'-sulphatophosphate sulphotransferase in *Chlorella*) and must be reduced by fraction C before it can accept the sulpho group from adenosine 3'-phosphate 5'-

sulphatophosphate (or adenosine 5'-sulphatophosphate). This mechanism predicts that fraction C is only required in catalytic amounts in the presence of its reducing system: enzyme A and NADPH. This mechanism would also explain the need for a cofactor for the oxidation of NADPH by enzyme A and purified fraction C (Asahi *et al.*, 1961). Although the earlier experiments have not been repeated, it seems likely that the cofactor that was observed in crude fraction C might have been the oxidized carrier, i.e., the protein precursor of protein-SSO₃⁻. At that time it seemed that oxidized fraction C was required in substrate amounts. More likely, as shown in Scheme 1, oxidized carrier was being supplied in substrate amounts in the crude fraction C preparation. This conclusion is supported by the present results showing stimulation of protein-SSO₃⁻ formation by the addition of crude fraction C.

The enzyme A/NADPH reducing system can be replaced by reduced lipoate (Hilz & Kittler, 1960; Wilson *et al.*, 1961), or by dithiothreitol as shown here, but only when fraction C is present. Thus fraction C is an obligatory intermediate between an exogenous supply of dithiol and the formation of exchangeable sulphite from adenosine 3'-phosphate 5'-sulphatophosphate in yeast. Further, reduced fraction C is the immediate H donor since oxidized fraction C is inactive and the reduction of fraction C by reduced lipoate and dithioreitol has been demonstrated experimentally in the present study (Table 2) and by Asahi *et al.* (1961).

It is possible that the thiols needed in the *Chlorella* system may function by reducing catalytic amounts of endogenous fraction C. Alternatively, the large amounts of thiols used in the *Chlorella* system may have carried out non-specific reductions. In either case it seems likely that the enzyme A-fraction C reducing system may operate *in vivo* in *Chlorella*.

General discussion

Although adenosine 5'-sulphatophosphate is the primary substrate for reduction in *Chlorella* (Schiff & Hodson, 1973) and spinach (Schmidt, 1975), the available data suggest that adenosine 3'-phosphate 5'-sulphatophosphate is the substrate in these studies with yeast enzymes. Adenosine 5'-sulphatophosphate was not apparent in the radioactivity scans of the electrophoresis strips, F⁻ was present to inhibit Mg²⁺-activated 3'-nucleotidase activity, and the omission of Mg²⁺ resulted in a stimulation rather than an inhibition of exchangeable sulphite formation. It was shown by Wilson *et al.* (1961) that adenosine 3'-phosphate 5'-sulphatophosphate was at least twice as effective as adenosine 5'-sulphatophosphate in the formation of exchangeable sulphite. Other reports indicate that adenosine 3'-phosphate 5'-sulphatophosphate is reduced in *E. coli* (Tsang &

Schiff, 1974) and spinach (Burnell & Anderson, 1973).

The exchange and binding studies that have been used to characterize the various forms of bound sulphite support the hypothesis that the sulpho group of adenosine 3'-phosphate 5'-sulphatophosphate and adenosine 5'-sulphatophosphate is transferred to a protein thiol group with the formation of an -S-SO₃⁻ linkage. Other linkages are not yet ruled out (Schmidt *et al.*, 1974) and additional studies, such as isolation and characterization of the thiol-containing component of the carrier peptide, are needed to verify the nature of this linkage. If the product is identified as an organic thiosulphate (Bunte salt), enzyme B of yeast and adenosine 5'-sulphatophosphate sulphotransferase of *Chlorella* should be termed S-sulphotransferases to distinguish them from the O- and N-sulphotransferases previously described (Roy & Trudinger, 1970; Jansson *et al.*, 1975).

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